

Promoter for the epidermis-specific transgenic expression in plants

The present invention relates to promoter regions, under whose control transgenes can be epidermis-specifically expressed in plants. Furthermore, the invention relates to recombinant nucleic acid molecules, which comprise said promoter regions, and to transgenic plants and plant cells, which have been transformed by means of said nucleic acid molecules, as well as to methods for their generation. Furthermore, the present invention relates to nucleic acid molecules comprising a promoter according to the present invention, and to nucleic acid sequences or transgenes, which are capable of mediating pathogen resistance, as well as to plants and plant cells transformed by means of said nucleic acid molecules, and to methods for their generation.

Those DNA regions of a gene, which are located upstream of the transcription initiation point and by which the initiation point and the initiation frequency of the transcription and thus the expression level and the expression pattern of the controlled gene are determined, are in general referred to as promoters. RNA polymerase and specific transcription factors activating the RNA polymerase bind to the promoters in order to initiate transcription together with the basal transcription complex. The effectiveness of the promoters is often enhanced and regulated by additional DNA sequences, the enhancer sequences, whose position, contrarily to the position of the promoters, is not fixed. These regulatory elements can be located upstream, downstream, or in an intron of the gene to be expressed.

In recombinant DNA technology, promoters are inserted into expression vectors in order to control the expression of a transgene, which is normally not the gene naturally regulated by the promoter. Of substantial significance herein is the specificity of the promoter, which determines at which point in time, in which types of tissue, and at which intensity a gene transferred by means of genetic engineering is expressed.

In plant breeding, recombinant DNA technology is often used for transferring specific advantageous properties to useful plants, which is supposed to lead to a higher yield, for example by means of increased pathogen resistance, or to improved properties of the harvest products. Herein, it is often desirable that the transferred gene be not expressed ubiquitously,

but only in those tissues, where the transgenic activity is desired, as the presence of the transgenic product can have a negative effect on normal physiological processes in some tissues. Thus, it could, for example, be shown that the overexpression of an anionic peroxidase under the control of the ubiquitously effective 35S promoter leads to wilting of transgenic tobacco plants, as less root growth occurs and therefore also less root mass is developed (Lagrimini et al. (1997) The consequence of peroxidase overexpression in transgenic plants on root growth and development. *Plant Mol Biol.* 33 (5), S. 887-895). The overexpression of the spi2 peroxidase under the control of the likewise ubiquitously effective ubiquitin promoter leads to reduced epicotyl development and reduced longitudinal growth in comparison with control plants (Elfstrand, M. et al. (2001) Overexpression of the endogenous peroxidase-like gene spi2 in transgenic Norway spruce plants results in increased total peroxidase activity and reduced growth. *Plant Cell Reports* 20 (7), S. 596-603). Irrespective of negative effects on physiological processes, it is often supposed to be prevented in resistance breeding that the transgenic product is also present in the harvested plant parts.

Therefore, promoters functioning either tissue-specifically or inducibly have been isolated during the past years. Tissue-specific promoters are, for example, seed-, tuber-, and fruit-specific promoters. The inducible promoters can be activated, for example, by means of chemical induction, light induction, or other stimuli.

It is also desirable to specifically modulate gene expression in the epidermis. The epidermis is the terminal tissue of the above-ground organs of higher plants. As such, the tasks of the epidermis are, on the one hand, to allow water and nutrient exchange of the plant and, on the other hand, to prevent the intrusion of pathogens into the plant. These functions could be specifically modulated by means of altered gene expression in the epidermis with the aid of suitable promoters and genes controlled by the latter.

Epidermis-specific promoters have already been described in dicotyledonous plants. It could thus be shown that the promoter of the CER6- (CUT1-) gene from *Arabidopsis*, which codes for a condensing enzyme in wax synthesis, can cause the epidermis-specific expression of a β -glucuronidase reporter gene (Hooker et al. (2002), Significance of the expression of the CER6 condensing enzyme for cuticular wax production in *Arabidopsis*, *Plant Physiol.* 129(4), S. 1568-1580; Kunst et al. (2000), Expression of the wax-specific condensing enzyme CUT1 in *Arabidopsis*, *Biochem. Soc. Trans.* 28(6), S. 651-654).

However, suitable epidermis-specific promoters in monocotyledonous plants, which are particularly well suitable for the expression of transgenes in monocotyledons, in particular in poaceae (sweet grasses), could not successfully be identified up to now. Therefore, constitutive promoters like the ubiquitin promoter from maize were hitherto used in order to express proteins in the epidermis (see, for example, Oldach et al. (2001), Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat, *Mol Plant Microbe Interact.* 14(7), S. 832-838). However, this can lead to undesired side effects in the transgenic plants due to the presence of the transgenic product in other tissues or organs than the epidermis, as is described above.

It is therefore the problem underlying the present invention to provide means allowing an epidermis-specific gene expression in monocotyledons, preferably in cereal plants.

This problem is solved by provision of the embodiments characterized in the patent claims.

Thus, the present invention relates to a promoter region having specificity for the plant epidermis, comprising a first sequence originating from the promoter of the gene glutathione-S-transferase A1 (GSTA1) and a second sequence originating from the intron of the gene WIR1a. GSTA1 relates to genes as they are described in Dudler et al. (1991), A pathogen-induced wheat gene encodes a protein homologous to glutathione-S-transferases, *Mol. Plant Microbe Interact.* 4(1), S. 14-18. In particular, these genes are genes from wheat; they can, however, also be homologous genes from other cereal plants, in particular from barley, having a comparable expression pattern and a similar-gene-product. WIR1a denotes genes as they are described in Bull et al. (1992), Sequence and expression of a wheat gene that encodes a novel protein associated with pathogen defense, *Mol. Plant Microbe Interact.* 5(6), S. 516-519.

Preferably, the first sequence is SEQ ID No. 1 and the second sequence is SEQ ID No. 2.

Between the first and the second sequence there can be further non-translated sequences having a length of 10 bp to 1000 bp, preferably of 20 bp to 800 bp, particularly preferably of 30 bp to 500 bp, and most preferably between 40 bp and 300 bp.

Particularly preferably, the promoter region according to the present invention is a promoter region selected from the group consisting of

- a) promoter regions comprising the nucleic acid sequence given in SEQ ID No. 3;
- b) promoter regions comprising a functional part of the nucleic acid sequence given in SEQ ID No. 3 or
- c) promoter regions having a sequence, which hybridizes under stringent conditions with the nucleic acid sequence given in SEQ ID No. 3.

Within the scope of the present invention, a promoter region is understood to be a nucleic acid sequence comprising the regulatory sequences required for the expression of a coding sequence (transgene). Regulatory sequences form that part of a gene, which determines the expression of a coding sequence, i.e. in particular the expression level and pattern. The regulatory sequences have at least one sequence motif, where the specific transcription factors and the RNA polymerase bind, assemble to form the transcription complex, and effectively initiate the transcription of the nucleic acid sequence controlled by the promoter region.

The promoter regions according to the present invention are based on the observation that promoters having new properties can be generated by means of fusing the promoter of the GSTA1 gene from wheat with intron sequences of the WIR1a gene from wheat.

In transient reporter gene assays in wheat leaves having a β -glucuronidase (GUS) gene from *E. coli* as reporter gene, different combinations of the WIR1a promoter and intron and the GST promoter were tested. Surprisingly, it showed that GST promoter and WIR1a intron have a synergistic effect on reporter gene activity. The increase in transcriptional activity was comparable to the transcriptional activity achieved by means of the ubiquitously expressed 35S promoter.

Within the scope of the present invention, the term "epidermis-specific" is understood to denote that a nucleic acid sequence, which is under the control of the promoter region according to the present invention, is expressed in the shoot epidermis of plants. In the sense of the present invention, epidermis-specificity is, in particular, also given, if the promoter region according to the present invention favors the expression of a foreign gene in the epidermis in comparison with other cell types and causes a significantly increased, like at

least double, preferably at least 5-fold, particularly preferably at least 10-fold, and most preferably at least 50-fold, expression in comparison with other cell types. The expression level can be determined by means of conventional *in situ* detection techniques.

The term "plant epidermis" is known to the person skilled in the art. Complementary information can be found in any book on plant anatomy or plant physiology, like, for example, in Strasburger, Lehrbuch der Botanik, 35. edition 2002, Spektrum Akademischer Verlag.

It has now surprisingly be found, that a promoter region, which comprises both regulatory sequences from the GSTA1 gene from wheat and intron sequences from the WIR1a gene from wheat, causes epidermis-specific expression of a coding nucleic acid sequence, which is under its control.

Beside a promoter region having the nucleic acid sequences depicted in SEQ ID No. 3, the present invention also relates to promoter regions having functional parts of said sequence and causing epidermis-specific expression of one of the coding nucleic acid sequences, which they control, in plants.

In this context, a "functional part" is understood to denote sequences, which the transcription complex, despite a slightly deviating nucleic acid sequence, can still bind to and cause epidermis-specific expression. Functional parts of a promoter sequence also comprise such promoter variants, whose promoter activity is lessened or enhanced in comparison with the wild-type. In particular, a functional part is, of course, also understood to denote natural or artificial variants of the sequence of the promoter region given in SEQ ID No. 3. Mutations comprise substitutions, additions, deletions, exchanges, and/or insertions of one or more nucleotide residue/s. Within the scope of the present invention, functional parts of the promoter regions comprise naturally occurring variants of SEQ ID No. 3 as well as artificial nucleotide sequences, for example obtained by means of chemical synthesis.

In any case, the promoter used contains a TATA box (positions 2163 to 2169 in SEQ ID Nos. 1 and 3) and preferably also two CAAT boxes (positions 1047 to 1051 or 1895 to 1899 in SEQ ID Nos. 1 and 3). Furthermore, the promoter contains at least one, preferably

at least two and three, particularly preferably at least four, five, and six, and most preferably at least seven or eight of the following sequence motifs:

- a) GTGGGGG
- b) ACGTGGA
- c) TCCACCT
- d) TATCCAT
- e) CATGCATG
- f) TGTAAG
- g) CCTACCA
- h) AATAGTA

Preferably, the sequence motifs are located at the positions corresponding to the following positions in SEQ ID Nos. 1 and 3:

- a) 185-191 and 217-223 bp
- b) 455-461 bp
- c) 508-514 bp
- d) 564-570 bp
- e) 1514-1521 bp
- f) 1520-1526 bp
- g) 1569-1575 bp
- h) 1610-1616 bp

The promoter activity of variants of the promoter region can be measured with the aid of marker genes, whose coding sequence is under the control of the promoter region to be examined. Suitable marker genes are, for example, the β -glucuronidase (GUS) gene from *E. coli*, a fluorescence gene like, for example, the green fluorescence protein (GFP) gene from *Aequoria victoria*, the luciferase gene from *Photinus pyralis* or the β -galactosidase (lacZ) gene from *E. coli*. Absolute promoter activity is determined by means of comparison with a wild-type plant. Tissue or cell specificity can easily be determined by means of comparison of the expression rates of the above-mentioned marker genes in the respective tissues or cells.

The present invention also relates to promoter regions having a nucleic acid sequence hybridizing with the nucleic acid sequence given in SEQ ID No. 3 under stringent conditions. In the context of the present invention, the term "hybridization under stringent conditions" means that hybridization is conducted *in vitro* under conditions, which are stringent enough to ensure a specific hybridization. Such stringent hybridization conditions are known to the person skilled in the art and can be taken from the literature (Sambrook et al. (2001), Molecular Cloning: A Laboratory Manual, 3. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

In general, "specifically hybridize" means that a molecule preferentially binds to a specific nucleotide sequence under stringent conditions, if said sequence is present in the form of a complex mixture of (for example total) DNA or RNA. The term "stringent conditions" generally denotes conditions, under which a nucleic acid sequence will preferentially bind to its target sequence and to a considerably smaller extent or not at all to other sequences. Stringent conditions are partially sequence-dependent and will be different under different circumstances. Longer sequences specifically hybridize at higher temperatures. In general, stringent conditions are selected in such a way that the temperature lies about 5°C below the thermal melting point (T_m) for the specific sequence at a defined constant ionic strength and a defined pH value. T_m is the temperature (under defined ionic strength, pH value, and nucleic acid concentration), at which 50% of the molecules complementary to the target sequence hybridize to the target sequence in a state of equilibrium. Typically, stringent conditions are those, wherein the salt concentration is at least about 0.01 to 1.0 M sodium ion concentration (or any other salt) at a pH value of between 7.0 and 8.3 and the temperature is at least 30 °C for short molecules (i.e. for example 10 to 50 nucleotides). In addition, stringent conditions can be achieved by means of adding destabilizing agents, like for example formamide.

Suitable stringent hybridization conditions are, for example, also described in Sambrook et al., *vide supra*. Thus, hybridization can, for example, occur under the following conditions:

- hybridization buffer: 2 x SSC, 10 x Denhardt's solution (Fikoll 400 + PEG + BSA; ratio 1:1:1), 0,1% SDS, 5 mM EDTA, 50 mM Na_2HPO_4 , 250 µg/ml herring sperm DNA; 50 µg/ml tRNA or 0.25 M sodium phosphate buffer pH 7,2, 1 mM EDTA, 7% SDS at a hybridization temperature of 65°C to 68°C
- washing buffer: 0.2 x SSC, 0,1% SDS at a washing temperature of 65°C to 68°C

Preferably, such promoter variants have a sequence identity of at least 50%, preferably at least 70%, particularly preferably at least 90%, and most preferably at least 95% to the promoter sequence given in SEQ ID No. 3 or parts thereof, in relation to the total DNA sequence shown in SEQ ID No. 3. Preferably, the sequence identity of such promoter sequences is determined by means of comparison with the nucleic acid sequence given under SEQ ID No. 3. In case two nucleic acid sequences of different length are compared to each other, the sequence identity preferably relates to the percentage of the nucleotide residues of the shorter sequence, which are identical to the corresponding nucleotide residues of the longer sequence.

Sequence identities are conventionally determined via different alignment programs, like for example CLUSTAL. In general, the person skilled in the art has at his disposal suitable algorithms for determining the sequence identity, for example also the program, which is accessible under <http://www.ncbi.nlm.nih.gov/BLAST> (for example the link „standard nucleotide-nucleotide BLAST [blastn]“).

The percentage degrees of identity given above for SEQ ID No. 3 also apply to the first and second sequences of the promoter region according to the present invention, which are shown in SEQ ID Nos. 1 and 2.

In a preferred embodiment of the invention, the promoter region according to the present invention has the total sequence of 2552 nucleotides, which is given under SEQ ID No. 3.

The present invention also relates to chimeric genes of the promoter region according to the present invention and of a coding sequence, whose expression, which is naturally not regulated by the promoter region according to the present invention, in the chimeric gene is regulated by the promoter region according to the present invention, in operative linkage as well as to recombinant nucleic acid molecules containing said chimeric gene.

The term “nucleic acid sequence, whose expression is regulated by the promoter region according to the present invention” means that the expression of the nucleic acid sequence under the control of the promoter region according to the present invention in those cells, in which the promoter region is active, can be increased by at least the factor five, preferably at

least the factor 10, and particularly preferably at least the factor 50 in comparison with the wild-type cells.

The nucleic acid sequence, whose expression is regulated by the nucleic acid sequence according to the present invention, can be the coding region of a transgene, for example a resistance gene, whose gene product is desired in the epidermis. By means of expression of the transgene, the content of the gene product encoded by it can be increased by at least the factor 2, preferably by at least the factor 5, particularly preferably by at least the factor 10, and most preferably by at least the factor 50.

However, the promoter region according to the present invention can also be used in RNAi constructs for RNA interference in order to achieve the epidermis-specific silencing of specific genes, whose gene products are supposed to be present in the epidermis to a smaller extent than usual or not at all. Of course, the latter can also be achieved by means of classic antisense or co-suppression constructs with the use of the promoter region according to the present invention. By means of the silencing constructs, the expression of the endogenous gene is decreased by at least 50%, preferably by at least 70%, particularly preferably by at least 90%, and particularly preferably by at least 95%.

In a construct, which is supposed to be used for RNA interference, there are usually palindromic DNA sequences, which form double-stranded RNA subsequent to the transcription. By means of the dicer enzyme, said double-stranded RNA is processed to form shorter RNA pieces, which bind to an endogenous RNA and cause its degradation with the aid of the RISC (RNA-induced silencing complex) (Hannon (2002) RNA-interference, Nature, Bd. 418, S. 244-251).

The effect of the gene silencing constructs on the expression of the endogenous gene can be detected by means of conventional molecular biological methods, which are well known to the person skilled in the art. Thus, Northern blot and RT-PCR methods are available for examining the RNA level; the protein can be detected by means of Western blot analyses, immunofluorescences, or, provided that the protein is an enzyme, by means of enzyme assays.

Within the scope of the present invention, the term “transgene” summarizes those genes, whose gene products are supposed to be provided in the epidermis or are supposed to be suppressed in gene silencing.

Preferably, the nucleic acid sequence, whose expression is under the control of the promoter according to the present invention, is a nucleic acid sequence, which mediates pathogen resistance, as the epidermis is the first band, which has to be surmounted by a pathogen when intruding into the plant.

Within the scope of the present invention, the term “recombinant nucleic acid molecule” is understood to denote a vector, which contains a chimeric gene according to the present invention or a promoter region according to the present invention and which can cause the promoter-dependent expression of the nucleic acid sequence; which is under the control of the promoter region according to the present invention, in plant cells and plants. In a preferred embodiment, a recombinant nucleic acid molecule according to the present invention additionally contains transcription termination sequences. Herein, “transcription termination sequences” are understood to denote DNA sequences, which are located at the downstream end of a coding sequence and which cause the RNA polymerase to terminate the transcription.

Furthermore, the invention relates to methods for generating transgenic plants with epidermis-specific expression of a nucleic acid sequence, which is regulated by the promoter region according to the present invention, comprising the following steps:

- a) generating a recombinant nucleic acid molecule, in which the promoter region according to the present invention is present in operative linkage with a coding sequence,
- b) transferring the nucleic acid molecule from a) to plant cells and
- c) regenerating entirely transformed plants and, if desired, propagating the plants.

For the preparation of the introduction of foreign genes into higher plants and their cells, respectively, a large number of cloning vectors containing a replication signal for *E. coli* and a marker gene for selecting transformed bacteria cells are available. Examples for such vectors are pBR322, pUC series, M13mp series, pACYC184, and so on. The chimeric gene can be introduced into the vector at a suitable restriction site.

The plasmid obtained is then used for transforming *E. coli* cells. Transformed *E. coli* cells are cultivated in a suitable medium and are subsequently harvested and lysed and the plasmid is re-obtained. Restriction analyses, gel electrophoreses, and further biochemical-molecular biological methods are generally used as analysis methods for characterizing the obtained plasmid DNA. Subsequent to each manipulation, the plasmid DNA can be cleaved and DNA fragments obtained therefrom can be linked with other DNA sequences.

As already mentioned, a variety of techniques for introducing DNA into a plant host cell are available, wherein the person skilled in the art can determine the method suitable in each case without any difficulties. Said techniques comprise transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation medium, fusion of protoplasts, injection, electroporation, direct gene transfer of isolated DNA into protoplasts, introduction of DNA by means of biolistic methods as well as further possibilities, which are well established for several years now and which belong to the standard repertoire of the person skilled in the art of plant molecular biology and plant biotechnology, respectively. The biolistic gene transfer method is, in particular, used in monocotyledonous plants. Here, the person skilled in the art can find useful information on the conduction, like for example in Vasil et al. (1992) Bio/Technology, 10, S. 667-674; Vasil et al. (1993) Bio/Technology, 11, S. 1153-1158; Nehra et al. (1994) Plant J. 5, S. 285-297; Becker et al. (1994) Plant J., 5, S. 299-307; Altpeter et al. (1996) Plant Cell Reports 16, S. 12-17; Ortiz et al. (1996) Plant Cell Reports 15, S. 877-81; Rasco-Gaunt et al. (2001) J. Exp. Bot. 52; S. 865-874.

In the case of injection and electroporation of DNA into plant cells, no specific demands per se are made on the plasmids used. This also applies to direct gene transfer. Simple plasmids, like for example pUC derivatives, can be used.

However, if whole plants are supposed to be regenerated from cells transformed in this manner, the presence of a selectable marker gene is recommendable. Standard selection markers are known to the person skilled in the art and selecting a suitable marker does not pose a problem.

According to the method of introducing the desired genes into the plant cell, further DNA sequences can be required. If, for example, the Ti or Ri plasmid is used for transforming the plant cell, at least the right border, though often the right and left border, of the T-DNA contained in the Ti or Ri plasmid, have to be joined with the genes, which are supposed to be introduced, to form a flanking region. If agrobacteria are used for transformation, the DNA, which is supposed to be introduced, has to be cloned into specific plasmids, actually either into an intermediate or into a binary vector. Due to sequences, which are homologous to sequences in the T-DNA, the intermediate vectors can be integrated into the Ti or Ri plasmid of the agrobacteria by means of homologous recombination. Said plasmid also contains the vir region necessary for the transfer of the T-DNA. However, intermediate vectors cannot replicate in agrobacteria. By means of a helper plasmid, the intermediate vector can be transferred to *Agrobacterium tumefaciens* (conjugation). Binary vectors, however, can replicate in both *E. coli* and in agrobacteria. They contain a selection marker gene and a linker or polylinker, which are framed by the right and left T-DNA border region. They can be transformed directly into the agrobacteria. The agrobacterium serving as a host cell should contain a plasmid carrying the chimeric gene within the T-DNA, which is transferred into the plant cell. Additional T-DNA can be present. The agrobacterium transformed in such a way is used for the transformation of plant cells. The use of T-DNA for the transformation of plant cells has been intensely examined and sufficiently described in commonly known survey articles and manuals on plant transformation. In the case of monocotyledonous plants, altered protocols must be applied for effective agrobacterium-mediated gene transfer, as they are, for example, described in Cheng et al. (1997) Plant Physiol. 115, S. 971-980; Khanna and Daggard (2003) Plant Cell Reports 21, S. 429-436; Wu et al. (2003) Plant Cell Reports 21, S. 659-668; Hu et al. (2003) Plant Cell Reports 21, S. 1010-1019. For the transfer of the DNA into the plant cell, plant explants can advisably be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Whole plants can then be regenerated from the infected plant material (e.g. pieces of leaves, segments of stems, roots, but also protoplasts or suspension-cultivated plant cells) in a suitable medium, which can contain antibiotics or biocides for the selection of transformed cells.

Once the introduced DNA is integrated in the genome of the plant cell, it is normally stable there and is also maintained in the offspring of the originally transformed cell. The introduced DNA normally contains a selection marker, which mediates resistance against a biocide or an

antibiotic like kanamycin, G 418, bleomycin, hygromycin, methotrexate, glyphosate, streptomycin, sulfonylurea, gentamycin or phosphinotricin and others to the transformed plant cells. The individually selected marker should therefore allow the selection of transformed cells against cells lacking the introduced DNA. To this end, alternative markers, like nutritive markers or screening markers (like GFP, green fluorescent protein), are also suitable. Selection markers can, of course, also be entirely omitted, which, however, is accompanied by a comparatively high screening necessity. In case marker-free transgenic plants are desired, the person skilled in the art has also at his disposal strategies, which allow removing the marker gene later on, for example co-transformation or sequence-specific recombinases.

Regeneration of the transgenic plants from transgenic plant cells is conducted according to conventional regeneration methods using known nutritive media. The plants obtained in this manner can then be examined by means of conventional methods, including molecular biological methods like PCR, blot analyses for presence and tissue specificity of the introduced nucleic acid sequence, whose expression is controlled by the promoter according to the present invention, or for endogenous RNAs and proteins influenced by said nucleic acid sequence.

Furthermore, the invention relates to transgenic plants containing a nucleic acid sequence regulated by the promoter region according to the present invention and epidermis-specifically expressing said nucleic acid sequence.

Preferably, the plants according to the present invention are monocotyledons, in particular cereal plants like rye, maize, and oats, particularly preferably wheat or barley, as well as transgenic parts of said plants and their transgenic propagation material, like protoplasts, plant cells, calli, seeds, tubers or cuttings, as well as the transgenic offspring of said plants. However, the promoter region according to the present invention can also be used in other poaceae (sweet grasses), like for example feed grasses, for generating corresponding plants having epidermis-specific expression of transgenes.

Genes for the production of epicuticular waxes can also be expressed under the control of the epidermis-specific promoter according to the present invention in order to increase drought tolerance of the plants. In addition, genes for the production of anthocyanins or other UV-

absorbing substances for increasing UV-resistance can also be expressed under the control of the promoter according to the present invention. As was already worked out in the above, pathogen resistance genes are preferably expressed under the control of the promoter according to the present invention.

Bacteria, viruses, and fungi, which infect plants and thereby negatively influence the metabolism of the plant, are, inter alia, referred to as plant pathogens.

Among these plant pathogens are fungi, which, inter alia, cause the diseases mildew and stem break in cereal plants like wheat and barley. Depending on the degree of infection, these diseases can cause considerable yield losses (up to 50%).

Traditionally, the above-mentioned and further fungal plant diseases are controlled by means of fungicides, which have the known disadvantages, like percolation into groundwater and accumulation in the food chain.

Over the last few years, however, several genes, which are capable of mediating resistance against a specific agent or against several agents, were identified. The term "mediation of pathogen resistance", as it is used herein, means that plants, in which the expression of said genes is increased, are less susceptible for infections with specific pathogens in comparison with plants, in which the expression of said genes is normal. Among the genes, which mediate pathogen resistance, are also such genes, whose expression is activated by infection with a pathogen.

Among these genes are peroxidases and oxalate oxidases. The oxalate oxidases, which belong to the family of the germin-like proteins, catalyze the oxidation of oxalate, whereby hydrogen peroxide is formed. Hydrogen peroxide acts microbicidally and can enhance the lignification of the cell walls, whereby the intrusion of pests is prevented. Moreover, it can cause hypersensitive cell death at low concentrations. The peroxidases use either molecular oxygen or hydrogen peroxide in order to oxidize and thereby detoxify cellular substrates.

Pathogens, against which the expression of the oxalate oxidases and peroxidases in the epidermis of plants can mediate resistance, for example comprise: mildew, fusarium spp.,

rynchosporium secalis and *pyrenophora teres*. Further genes, which are capable of mediating resistance against pathogens, are chitinases, Ag-AFP, GSTA1, and WIR1a.

By means of expressing the nucleic acid sequence coding for said enzymes in the epidermis of transgenic plants with the aid of the promoter region according to the present invention, plants having increased pathogen resistance can be obtained.

In contrast to the genes mediating pathogen resistance, there are also plant-inherent genes, which promote the intrusion of a pathogen. Among those is the Mlo gene, which codes for a seven transmembrane receptor, which seems to promote the intrusion of the mildew fungus into the epidermis. In this case, it is appropriate to interfere with the expression of the Mlo gene in order to prevent the intrusion of fungi into the plant. This can, for example, be conducted with the aid of the above-described RNAi method. The fact that the interference with the expression of the Mlo gene is suitable for preventing the intrusion of the mildew fungus into the plant was shown *in vitro* in leaf segments from barley, which were bombarded with tungsten particles, which had been coated with Mlo-dsRNA (Schweizer et al. (2000), Double-stranded RNA interferes with gene function at the single-cell level in cereals, The Plant Journal, 24 (6), S. 895-903). However, it could hitherto not be shown that the epidermis-specific interference with the Mlo expression in transgenic plants has the same effect.

Further plant genes, which mediate the interaction of a pathogen with the plant and can thereby promote the intrusion of the pathogen into the plant, are, for example, amino acid or sugar transporters or invertases. Said genes are also suitable as targets for gene silencing. Thus, the present invention relates to methods for generating pathogen-resistant plants, comprising the steps:

- a) generating a recombinant nucleic acid molecule, in which the promoter according to the present invention is present in operative linkage with a nucleic acid sequence mediating pathogen resistance,
- b) transfer of the recombinant nucleic acid molecule from a) to plant cells and
- c) regenerating entirely transformed plants and, if desired, propagating said plants.

Preferably, the nucleic acid sequence mediating pathogen resistance is the coding region of a peroxidase or oxalate oxidase gene or a sequence, which interferes with the endogenous Mlo-RNA.

The following Examples serve for illustrating the invention and are not supposed to be understood as limiting.

Figures:

- 1) nucleic acid sequence of the GSTA1 promoter (SEQ ID No. 1)
- 2) nucleic acid sequence of the WIR1a intron (SEQ ID No. 2)
- 3) nucleic acid sequence of the preferred promoter region (SEQ ID No. 3)
- 4) nucleic acid sequence of the TAPERO (peroxidase) cDNA (SEQ ID No. 4)
- 5) TAPERO expression vector pPS41
 - a) nucleic acid sequence (SEQ ID No. 5)
 - b) vector map
- 6) nucleic acid sequence of the germin 9f-2.8 (oxalate oxidase) cDNA (SEQ ID No. 6)
- 7) germin expression vector pPS24
 - a) nucleic acid sequence (SEQ ID No. 7)
 - b) vector map
- 8) sequence of the Mlo-RNAi construct (SEQ ID No. 8)
- 9) Mlo-RNAi expression vector pWIR5-TaMlo RNAi.
 - a) nucleic acid sequence (SEQ ID No. 9)
 - b) vector map

10) *In situ* oxalate oxidase activity in pPS24 transgenic plants

Leaves from Bobwhite wild-type plants (BW) and from transgenic lines No. 157 and No. 170 were crosscut and the oxalate oxidase activity was detected *in situ*. Left column = reaction with oxalate substrate; right column = control reaction without oxalate substrate. The intense violet coloring indicates oxalate oxidase activity in the epidermis of the transgenic lines.

11) Detection of the TAPERO transgene in pPS41 transgenic plants

a) in the Northern blot

Detection of the accumulation of TAPERO RNA by means of hybridization of a WIR3 sample to Northern blots from transgenic wheat lines of the T2 generation, which carry the pPS41 construct. In each case, 2 sublines of 4 selected lines plus wild-type (BW) were analyzed in the adult plant stage. Leaf 1 = flag leaf. Leaves 2 to 4 = increasingly older. The TaGer-4 probe hybridizes to a group of stress-induced wheat genes and was used for testing pleiotropic side effects of the TAPERO overexpression. No significant side effect was found. EtBr = Loading control of the gels, stained with ethidium bromide.

b) in the Western blot

Detection of the accumulation of the TAPERO protein by means of antibody reaction on Western blots of transgenic wheat lines of the T2 generation, which carry the pPS41 construct. The TAPERO transgenic product has the expected size of 31 kD. In Bobwhite, leaf 3, an increased basal activity of the TAPERO gene can be observed. Leaf 1 = flag leaf. Coomassie stain = loading control of the gels, stained with Coomassie blue R 250.

12) Detection of the epidermis-specific transgenic expression

A) by means of Northern blot analysis

Detection of the accumulation of oxalate oxidase (left) and TaPERO (right) mRNA in the leaf epidermis of transgenic plants, which carry the pPS24 or the pPS41 construct, by means of specific probes. W = RNA from whole leaf. E = RNA from leaf epidermis. EtBr = gel stained with ethidium bromide as loading control; 26S

RNA = subsequent hybridization of the blot with a probe against the 26S ribosomal RNA as loading control.

B) by means of real-time reverse PCR analysis

The concentration of the TaPERO mRNA in whole leaf and epidermis of the transgenic line No. 2013 (transformed with the construct pPS41) was determined. The data were normalized by means of the constitutively expressed control genes UBC (ubiquitin-conjugating enzyme) and GAPDH (glyceraldehyde phosphate dehydrogenase). The expression remaining in the whole leaf comes from the non-removed upper leaf epidermis and from the phloem (side activity of the promoter).

C) by means of real-time reverse PCR analysis

Wild-type plants (Bobwhite) and the transgenic lines No. 2013 and No. 2151 (transformed with the pPS41 construct) were analyzed in the adult plant stage. The promoter is strongly expressed, in particular, in leaves and spikes. In stems and roots, the transgene is expressed not at all or only weakly.

13) Examination of mildew resistance of pPS41 transgenic plants

The flag leaf of adult plants was cut away and inoculated with wheat mildew in a detached leaf assay together with Bobwhite wild-type plants. 7 days after inoculation, the mildew infection was evaluated. Mean values from 3 independent inoculation experiments with plants of the T2 and T3 generation are shown. Subline 2088/2 does not express any TAPERO and is not increased resistant. Mean value "non-silenced" = mean value of all lines except 2088/2 and all experiments.

14) Shoot growth of pPS41 transgenic plants

Plants of the T2 generation were sown together with Bobwhite wild-type plants and photographed in the adult plant stage.

15) Examination of the mildew resistance of pWIR5-TaMlo-RNAi transgenic plants

The flag leaf of adult plants of the T2 generation was cut away and inoculated with wheat mildew in a detached leaf assay together with Bobwhite wild-type plants. 7

days after inoculation, the mildew infection was evaluated. 2 sublines per line were tested in each case.

Examples:

In the following examples, molecular biological standard methods like *E. coli* transformation, restriction digestion, ligation, DNA extraction, PCR, etc., as they are known in the art, were conducted according to Sambrook et al. (2001), vide supra. For all PCR reactions, proofreading *Pwo* polymerase (Roche) was used.

1) Generation of the promoter construct from GSTA1 promoter and WIR1a intron (pPS18)

Generation was conducted in several steps via the following precursor constructs: pPS1, pPS3, pPS15. All constructs contained the GUS reporter gene, so that they could be tested directly in a transient assay.

pPS1:

A 1.9 kb promoter fragment of the WIR1a gene was cut out of a recombinant pBluescript clone by means of *Pst*I and cloned into the *Pst*I restriction site of an expression cassette before the GUS gene. The expression cassette was based on pBluescript and contained the GUS gene followed by the transcription terminator of the wheat GSTA1 gene. As the GUS gene and the GSTA1 transcription terminator are no longer contained in the final constructs used (see Example 2), a detailed description of this expression cassette is omitted. The resulting construct contained a translational WIR1a::GUS fusion.

pPS3:

With the adaptor primers 5' ATA TAT CTG CAG GGA GCC ACG GCC GTC CAC and 5' TAT CCC GGG CCC GTG CCT GGA CGG GAA, a PCR fragment of about 240 bp was generated and its ends were cut with *Sma*I and *Pst*I (via Adaptor). The genomic WIR1a clone served as PCR template. The PCR fragment contained the last 15 amino acids of the first exon of WIR1a and the intron including splice site acceptor, and was ligated in pPS1, cut with *Pst*I (partially) and *Sma*I and purified by means of agarose gel electrophoresis. The resulting construct contained a translational WIR1a::GUS fusion with the WIR1 intron before the GUS

gene. Furthermore, a deletion of amino acids Nos. 18 - 35 of the first exon of WIR1a was introduced in order to prevent the secretion of the WIR1a::GUS fusion protein (by means of removing the signal peptide).

pPS15:

The WIR1a promoter was replaced by a PCR fragment of the GSTA1 promoter. To this end, pPS3 was (partially) digested with *Xho*I and *Sna*BI and the vector band was purified by means of agarose gel electrophoresis. The GSTA1 promoter fragment of about 2.3 kb length was amplified by means of PCR with the adaptor primers 5'ATA TAT CTC GAG TCT AGA ACT AGT GGA TCC and 5'ATA TAT TAC GTA GTT TGT CCG TGA ACT TCA from the genomic GSTA1 clone and cut at the ends with *Xho*I und *Sna*BI. The PCR fragment was ligated with the gel-eluated pPS3 band, resulting in a translational fusion of the intron-containing WIR1a gene fragment with GUS under the control of the GSTA1 promoter.

pPS18:

pPS15 was (partially) digested with *Pst*I and *Sna*BI, the vector band was purified by means of agarose gel electrophoresis and ligated with a double-stranded oligonucleotide (5'GTA CAC AGG CAG CTA GCT CTC GAA ACC TCG CTC GAA ACG CA plus 5'CAT GTG TCC GTC GAT CGA GAG CTT TGG AGC GAG CTT TGC GT). This replaced the part of the WIR1a gene located around the translation start (46 bp upstream to 53 bp downstream of the translation start) with 42 bp of the 5'UTR of the WIR1a gene without the translation initiation codon ATG. The resulting construct contained a transcriptional fusion of the intron-containing WIR1a gene fragment with GUS under the control of the GSTA1 promoter.

2) Generation of the constructs used

- a) expression vector pPS24 (oxalate oxidase expression under the control of the promoter according to the present invention)

A *Hind*III/*Sph*I fragment of 745-bp length of the wheat gf-2.8 gene (oxalate oxidase; Acc. No. M63223) containing the entire open reading frame (ORF) was subcloned into the plant expression cassette pGY1, which resulted in the construct pGermin (described in Schweizer et al., 1999). For this cloning, the oxalate oxidase fragment was ligated into an intermediate vector in order to be able to ligate the fragment by means of the restriction sites *Bam*HI and *Pst*I in pGY1.

From pGermin, a *SmaI/EcoRI* fragment of about 1 kb length, which contained the oxalate oxidase gene and the CamV 35S terminator, was ligated into the vector pPS18, which was *SmaI/EcoRI*-cut and purified by means of agarose gel electrophoresis. The resulting construct contained a transcriptional fusion of the intron-containing WIR1a gene fragment with the oxalate oxidase gene under the control of the GSTA1 promoter. Compared to pPS18, the construct did no longer contain the GSTA1 transcription terminator, but the transcription terminator of the CamV 35S gene.

b) expression vector pPS41 (TAPERO expression under the control of the promoter according to the present invention)

From pWIR3 (containing a transcriptional fusion of the CamV 35S promoter and TAPERO; Schweizer et al., 1999), a TAPERO fragment of about 1.2 kb length was isolated using *SmaI* and *PstI* by means of restriction digestion.

The TAPERO fragment was ligated in vector pPS24, which was (partially) digested with *SmaI* and *PstI* and was purified by means of agarose gel electrophoresis. This resulted in a transcriptional fusion of the intron-containing WIR1a gene fragment with the TAPERO gene (Acc. No. X56011) under the control of the GstA1 promoter, in which the oxalate oxidase gene was substituted by the TAPERO gene. Like pPS24, pPS41 contains the transcription terminator of the CamV 35S gene.

c) expression vector pWIR5-TaMlo-RNAi (expression of the Mlo-RNAi construct under the control of the promoter according to the present invention)

First, the third intron of the *Mlal* resistance gene from barley (about 1.1 kb), which was subcloned in the vector pGEM Teasy, was isolated by means of *EcoRI* and *PstI* and was ligated into the vector pBSw41 (pBluescript derivative with partial *TaMlo1* cDNA, cloned by Candace Elliott within the scope of her dissertation; GenBank accession No. AF361933), which was also *EcoRI*- and *PstI*-cut.

From this construct, the *Mlal* intron together with a part of the coding sequence of the *TaMlo1* gene was isolated as an about 1.55 kb *PstI/MscI* fragment (= fragment 1). Parallel to this, a fragment of about 450 bp was amplified by

means of PCR from the plasmid pBSw41 with the oligonucleotides T3 (standard sequencing primer for pBluescript) and TaMlo1-1 (5' GTC GCA TGC CTG TCC ACA CGA AAT GTG C 3', *Sph*I, restriction site underlined). Subsequently, the PCR fragment was digested by means of the restriction enzymes *Pst*I and *Sph*I (= fragment 2). The vector pPS24 (promoter + oxalate oxidase, see above) was opened by means of restriction digestion with *Sma*I and *Sph*I and the oxalate oxidase gene fragment, which was cut out, was discarded. Thereupon, the above-described fragments 1 and 2 were ligated into the *Sma*I/*Sph*I-cut vector pPS24 in a three-component ligation. In this ligation, the ends of the *Msc*I and *Sma*I-cut components are compatible, as both are so-called blunt ends. The resulting construct (pTaMlo1 RNAi) contains about 300 bp of the *TaMlo1* gene as well as about 150 bp polylinker/adaptor sequence as „inverted repeats“, separated by the *Mla*I intron. The control of this transcription unit is subject to the GSTA1 promoter.

Annotation: The gene herein referred to as *TaMlo1* for historical reasons was later named *TaMloA1* (Elliott *et al.*, 2002). Mol. Plant Microbe Interact. 15: 1069-1077 (2002).

3) Transformation of the wheat plants

Wheat plants (cv. Bobwhite) were raised in phytochambers for 40 days at 15°C during daytime and 12°C during nighttime under short day conditions (10 h/d, about 600 µE) and subsequently in a greenhouse at 18/16°C and a photoperiod of at least 16 h. The spikes were either used immediately or stored for up to 5 days at 4°C. The caryopses taken from the spike were surface-sterilized for 2 minutes with 70% ethanol and then for 15 to 20 minutes in 5% sodium hypochlorite solution / 0.1% Tween 20 and finally washed four times with sterile aqua bidest.

Unripe embryos having a size of 0.5 to 1.5 mm were prepared out of the caryopses under sterile conditions and were laid out on callus-inducing medium in petri dishes with their scutellum facing upward (basic medium according to Murashige Skoog (1962) with 2 mg/l 2,4-D, 40 g maltose monohydrate, 500 mg/l L-glutamine, 100 mg/l casein hydrolysate, 5 µM CuSO₄ and 0.25% phytagel). The cultures were incubated in the dark at 25°C.

The biolistic transformation was conducted five to seven days after isolating the embryos. Four to six hours prior to particle bombardment, the already proliferating embryos were transferred to a new medium having reduced water potential (as above, supplemented with 0.3 M mannitol) and incubated in the dark at 25°C.

The plasmid pAHC20 (Christensen and Quail 1996), which contains the *bar*-gene encoding phosphinothricin acetyltransferase, was mixed in a molar ratio of 1:1 with a vector to be co-transformed. Altogether, 10 µl plasmid DNA solution were then precipitated onto the particles of 25 µl of a 60 mg/l gold suspension. For one bombardment, 30 µg particles in 5 µl ethanol were applied onto a micro carrier. Bombardment was conducted according to the specifications of the manufacturer of the DuPont PDS-1000/He.

Twelve to 16 hours after particle bombardment, the explants were transferred to new callus-inducing medium (as for the pre-culture of the embryos) and incubated for 10 days in the dark at 25°C.

The calli were then transferred to differentiation medium (basic medium according to Murashige and Skoog (1962) with 20 g/l sucrose, 5 µM CuSO₄, 0.25% phytigel and 3 mg/l bialaphos) and were incubated with a photoperiod of 16 h at 200 µE and 25°C.

After 2 weeks, the transfer of the non-browned calli to regeneration medium (basic medium according to Murashige and Skoog (1962) with 20 g/l sucrose, 0.25% phytigel and 4 mg/l bialaphos) and a further incubation with a photoperiod of 16 h at 200 µE and 25°C was conducted.

After another 2 weeks, the grown shoots were thinned out, transferred to culture tubes containing regeneration medium and further cultivated with a photoperiod of 16 h at 200 µE and 25°C.

Identification of transgenic regenerates was conducted by means of the PAT activity test of leaf extracts according to Spencer et al. (1990) or by means of amplifying transgene-specific sequences from genomic DNA of the candidate plants and/or Southern blot with the use of a corresponding probe.

Depending on the quality of the basic material, the transformation efficiency of the method amounted to 0.5 to 3 transgenic plants per 100 embryos cultivated.

4) *In situ* oxalate oxidase activity in plants having the pPS24 construct

Leaf segments of Bobwhite wild-type plants or of pPS24 transgenic wheat plants of the T3 generation were infiltrated in vacuum with oxalate oxidase detection solution (2.5 mM oxalic acid, 3.5 mM free EDTA, 0.6 mg/ml 4-chloro-1-naphthol, 50 µg/ml peroxidase from horseradish, 20% v/v ethanol, adjusted to pH 4.0 by means of Tris base) and incubated overnight at +37°C. After removal of the detection solution, the leaves were incubated for another 24 h at +4°C in H₂O. Subsequently, the leaves were manually crosscut into thin segments and microscopized. Phase contrast light microscopy was conducted in a Zeiss Axiophot at 100-fold magnification. Cells with oxalate oxidase expression have cell walls stained violet.

5) Detection of the TAPERO transgene in pPS41 transgenic plants by means of Northern blot analysis

Leaves of Bobwhite plants and of pPS41 transgenic plants of the T2 generation (about 1 g fresh weight in each case, FW), both in the flag leaf stage, were homogenized in liquid nitrogen until a fine powder formed. The powder was added to 3 ml RNA extraction buffer (0.5 M Tris Cl pH 8.0; 0.25 M Na-EDTA; 5% (w/v) SDS) and 1.5 ml buffer-saturated phenol (15 ml plastic tubes) and well shaken. The extracts were centrifuged for 30 min at 4000 rpm - 5000 rpm, 20°C (swing out, Heraeus Varifuge). 1.5 ml chloroform were added (without draining the supernatant) and the tube was inverted several times. The extracts were re-centrifuged for 30 min at 4000 rpm - 5000 rpm, 20°C, and the supernatant was carefully poured into a new tube (15 ml plastic tube). The RNA was precipitated by means of adding 3 ml 6 M LiCl (overnight, 4°C). The precipitated RNA was centrifuged for 30 min at 12,500 rpm, 4°C (fixed rotor, Hermle Z360K), the RNA pellets were taken up in 500-1000 µl 70% ethanol (RNA does not dissolve) and transferred to Eppendorf tubes. The samples were centrifuged for 10 min at 14,000 rpm, 4°C (fixed rotor, Eppendorf Centrifuge 5417R), and the supernatant was lifted off. The RNA pellets were dried for 5 min at 37°C, taken up in 100 µl to 200 µl TE, and dissolved for 5 to 10 min at -75°C. The denaturing agarose gel electrophoresis of the RNA in formaldehyde-containing gels and the transfer to nylon

membranes (Hybond N, Amersham) was conducted according to standard protocols (Sambrook et al., *vide supra*). 10 µg RNA were applied per sample.

Radioactive probe labeling with α ^{32}P -dCTP was conducted according to the random prime labeling method using a kit (Roche). Hybridization was conducted overnight at 65°C in CHURCH buffer (0.5 M Na phosphate pH 7.2; 1 % (w/v) BSA; 7 % (w/v) SDS; 1 mM Na₂EDTA). The blots were washed twice for 15 min in washing solution (0.1 x SSC; 0.1 % (w/v) SDS) at 65°C and subsequently exposed for 16 to 48 h against phosphorimager screens. The exposed screens were scanned by means of a phosphorimager device (FujiFilm FLA 3000) and exported as image files in TIFF format.

6) Detection of the TAPERO transgene in pPS41 transgenic plants by means of Western blot analysis

Leaf tips of Bobwhite plants and of pPS41 transgenic plants of the T2 generation, both in the flag leaf stage, were homogenized in IWF buffer (32 mM Na-phosphate; 84 mM citrate; pH 2.8; spatula tip polyvinylpolypyrrolidone). The homogenates were centrifuged for 15 min at 13,000 rpm and 4°C. The supernatants were mixed with 0.5 g/ml ammonium acetate and acid-soluble proteins were precipitated overnight at 4°C.

The proteins were centrifuged for 30 min at 13,000 rpm and 4°C. The protein pellets were taken up in 50 µl/g FG re-suspension buffer (50 mM Tris-Cl pH 7.5; 20% (v/v) glycerol). 5 µl 4-fold concentrated SDS sample buffer were added to 20 µl sample and the samples were mixed with (1-5 µl) saturated Tris solution until the color of bromphenol blue changed to blue. For each lane, 12.5 µl boiled sample were separated in denaturing SDS polyacrylamide gel electrophoresis (15% separating gel) according to a standard method using mini-gel equipment by Bio-Rad.

Subsequent to electrophoresis, the gels were either Coomassie-stained (as loading control) or transferred according to a standard method to a nitrocellulose membrane (blotted). According to a standard method, the membranes were incubated with a first polyclonal antibody (dilution 1:2000), which was directed against the Prx8 protein from barley (a protein homologous to TAPERO), followed by the second antibody (dilution 1:2000), which was directed against rabbit antibodies and to which alkaline phosphatase was coupled. The TAPERO protein bands were detected by means of localized alkaline phosphatase activity (BCIP/NBT staining solutions; prefabricated tablets (Roche)).

7) Detection of the epidermis-specific transgenic expression by means of Northern blot analysis and real-time PCR analysis

RNA extraction and Northern blot analysis were conducted as described in Example 5. Real-time PCR analysis was conducted by means of a LightCycler® device (Roche, Mannheim, Germany) according to the manufacturer's specifications.

8) Mildew resistance in pPS41 or pWIR5-TaMlo-RNAi transgenic plants

For the resistance test, adult pPS41 or pWIR5-TaMlo-RNAi transgenic wheat plants were used, which had been grown in the greenhouse and had a fully developed freshly grown flag leaf. Simultaneously grown wild-type plants cv. Bobwhite served as controls. The apical half of the flag leaf was cut off and spread on 0.5% (w/v) phytoagar, which was mixed with 20 ppm benzimidazole, in 20 x 20 cm large polycarbonate dishes. One transgenic subline (20 leaves each) plus Bobwhite wild-type (6 leaves each) was spread per dish. The leaf segments were inoculated with mildew spores in an inoculation medium by means of blowing spores of 4 strongly inoculated wheat leaves into the tower. After 5 min, the dishes were removed, sealed, and incubated at 20°C in indirect daylight. Seven days after inoculation, the mildew infection was evaluated using a class evaluation system (Schweizer et al., 1995). Resistance was calculated with reference to the control leaves located on each respective phytoagar plate.

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